TUMOR-SPECIFIC IMMUNITY TO CHEMICALLY INDUCED TUMORS

Evidence for Immunologic Specificity and Shared Antigenicity in Lymphocyte Responses to Soluble Tumor Antigens*

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Chemical carcinogen-induced tumors evoke resistance to rechallenge which is unique to the immunizing tumor and does not extend to syngeneic tumor cells in most systems which have been investigated (1–3). By contrast when in vitro techniques such as colony inhibition (4, 5) or microcytotoxicity (6, 7) are employed, similar families of syngeneic tumors reveal common or shared antigens as targets for tumor-immune lymphoid cells. As an approach to resolving this apparent paradox, we have used solubilized preparations from such tumors to induce tumor-specific resistance in vivo, and to assess in vitro reflections of tumor immunity at the cellular level.

The in vitro assay is based upon measuring proliferation induced in lymphoid cells from tumor-bearing or tumor-immune mice by KCl-solubilized membranes derived from families of syngeneic methylcholanthrene (MCA)[1]-induced fibrosarcomas. Kinetic parameters of the assay are explored in detail, and evidence is developed that the antigen-lymphoid cell interaction in vitro has immunologic specificity. The assay is used to explore the evolution of bearing transplanted tumors and the transplantation-resistant states which follow surgical tumor excision or immunization with the soluble tumor antigens. Both in vitro and in vivo data suggest the probable existence of multiple antigenic components on such tumor cells and that some, but not all, of these components are shared with syngeneic tumors.

Materials and Methods

Mice. Age-matched, 7- to 8-wk old female C57BL/6, C57BL/10, C57BL/10·BR and C57BL/10·D2, obtained directly from Jackson Laboratories, Bar Harbor, Maine, were used in all experiments.

Tumors. Fibrosarcomas were induced in each strain by injecting 0.5 mg MCA in 0.1 ml olive oil intramuscularly (i.m.) in four sites. The tumors which arose 6–12 wk later were frozen after a single

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[1] *Abbreviations used in this paper:* FCA, Freund's complete adjuvant; LN, lymph node; MCA, methylcholanthrene; PBL, peripheral blood leukocytes; and [^{3}H]TdR, [^{3}H]thymidine.

syngeneic passage, then carried in syngeneic female mice an average of four passages while being used to provide the experimental tumor inoculum. The tumors used in the experiments presented in this paper are in the C57BL/6 (C57-M1, C57-M2, C57-M3, C57-M4, $C_3$57-M1, and $C_3$57-M2) or C57BL/10·BR (B10·BR-M1, B10·BR-M2, and B10·BR-M3) lines. Tumor cell suspensions were prepared as previously described (8) and transplanted by i.m. inoculation into the distal thigh of 8-wk old mice.

Because in vitro and in vivo evidence for immunity to multiple or shared antigens was discovered in the course of these experiments, a convention for the tumor-host relationship terminology has been adopted for clarity. "Homologous" tumor refers to that specific tumor or soluble antigen derived from the tumor which the animal is bearing, or the tumor to which it has been immunized. "Syngeneic tumor," or the soluble products thereof, refers to all other MCA-induced tumors raised in that same strain or subline of mice.

Soluble Tumor Antigens. Soluble tumor antigens were prepared by modification of the method of Reisfeld et al. (9). All operations were conducted under strictly aseptic conditions and involved sterile pyrogen-free solutions. Carefully dissected fresh tumor tissue was suspended in sterile pyrogen-free phosphate-buffered saline containing 3 M KCl at pH 7.4 (2 ml solvent/1 g original tumor tissue weight) and briefly disrupted at 4°C by homogenization in a sterilized Sorvall blender (Ivan Sorvall, Inc., Newtown, Conn.). This suspension was stirred at 4°C for 16 h, then centrifuged at 225,000 *g* for an additional 60 min. The resulting supernatant preparations (hereafter called tumor antigen) were exhaustively dialysed against phosphate-buffered saline, diluted to a uniform level of absorbance at 280 nm, passed through a 0.2 μm millipore filter, and stored no longer than 3–4 wk at 4°C until used. The preparations are referred to in the text by the strain and a number designation followed by the bracketed suffix [s]; for example, B10-M4[s] is soluble antigen prepared from the M4 tumor raised in C57BL/10 mice. The solubilized tumor preparations were tested and found to be negative for pyrogenicity in rabbits by standard methods.

Cell Preparations. Spleen or lymph node (LN) cells were removed after exsanguination via the abdominal aorta, minced with scissors, and passed through sterile 60 mesh stainless steel screens using RPMI-1640 as the suspending medium. Cells were then freed from clumps by drawing the suspension through 25-gauge needles, and total cell counts were determined for each cell mass assayed.

Peripheral blood was collected from the retro-orbital plexus using heparinized tubes. A total and differential cell count was made, the blood was centrifuged at 2,000 rpm for 20 min, and the buffy coat and serum were collected in cold RPMI-1640. The pooled peripheral blood leukocyte (PBL) suspension was washed twice with cold PRMI-1640. The results were related to the absolute lymphocyte count.

Assay for Mitogen and Tumor Antigen Stimulation. The culture medium employed throughout consisted of RPMI-1640 to which 100 μg streptomycin/ml had been added together with 56°C heat-activated human serum. The culture method has been reported in detail in previous papers (9, 10). The antigens or mitogens were added to polypropylene culture tubes (12 × 75 mm, BioQuest Div., Becton, Dickinson & Co., Cockeysville, Md.) containing 1×10^6 cells, then cultured for 72 h in 5% CO_2 in air. Tumor antigens were added in the amounts indicated in each instance to cultures containing 0.5, 1×10^6, or 2×10^6 cells in 0.5 ml culture medium and incubated for 72 h. Dose-response titrations were performed in all assays; that dose giving maximal incorporation values is reported in some of the experiments to be described. [^{3}H]thymidine ([^{3}H]TdR) incorporation was assayed by the method previously reported (8, 10). The data obtained are presented as mean values ± standard error (SE) for four replicate cultures at each dose level.

Assay of Immunogenicity of Growing Tumors or Solubilized Tumor Antigens. The immunogenicity of each tumor was assayed initially by the classical transplantation technique in syngeneic recipients (11). Groups of five mice were injected with 1×10^5 viable tumor cells in the hind limb. 7 days later this limb was surgically excised; the mice were then challenged 7 days later with 10-fold incremental numbers of tumor cells (1×10^2–1×10^6) s.c. in the other hind limb. Tumor growth was assessed by weekly measurement of tumor sizes for 5 wk. Control animals did not receive a priming injection of tumor, but their hind limb was amputated at the same time as tumor bearers, and both were challenged with tumor on the same schedule.

In order to assay for immunogenicity of tumor antigen preparations, 0.1 mg KCl-solubilized tumor material was incorporated into Freund's complete adjuvant (FCA) and injected i.m. into groups of

five mice (12, 13). 4 wk later the injection was repeated s.c., and 1 wk after that groups of such animals, together with controls which had received only FCA, were challenged with incremental numbers of viable tumor cells. Tumor growth was measured at weekly intervals thereafter.

Results

Kinetics of Stimulation of Lymphoreticular Cells by Homologous Solubilized Tumor Preparations. To determine the optimal conditions for the in vitro stimulation of proliferation induced by 3 M KCl-solubilized MCA tumors, experiments were performed involving multiple variations in culture conditions, dose-response relationships, and time-course kinetics. The data presented in Table I and Figs. 1–3 are representative of kinetic data obtained in over 100 similar experiments done in various contexts. The correlation between the

TABLE I

*Time Course of [^{3}H]TdR Incorporation Stimulation by C57-M4[s] in Cultures of Spleen Cells from C57-M4-bearing C57BL/6 Mice**

Duration of cultures	[^{3}H]TdR incorporation	
	Control	C57-M4[s] added
h	*mean cpm ± SE/10^6 cells*	
24	32,109 ± 537	37,249 ± 559
48	2,888 ± 255	4,636 ± 293
72	2,533 ± 89	6,370 ± 149
96	218 ± 14	215 ± 7

* Cultures of 2×10^6 normal C57BL/6 or C57BL/6 M4-bearing spleen cells (21 days postinoculaton) were established, and graded amounts of C57BL/6 M4[s] were added; the cultures were terminated and [^{3}H]TdR incorporation (added 24 h earlier) was assayed. Data shown represent mean peak incorporation values ± SE.

kinetics of stimulation and the evolution of tumor immunity at the cellular level will be considered in a later section.

The typical time-course kinetics of solubilized antigen-induced proliferation in spleen cells taken from tumor-bearing mice are shown in Table I. Discrimination between spontaneous proliferation in unstimulated (control) cells from tumor-bearing animals and peak proliferation induced in those cultured with C57BL/6[s] was maximal at 72 h of culture. This culture interval was employed for all subsequent assays.

Dose-response titrations of cells taken from tumor-bearing hosts gave data which varied according to the population of cells assayed, the interval after tumor inoculation or amputation, and other factors. The range of proliferation behavior observed in PBL from both normal and tumor-bearing mice, in response to solubilized homologous tumor, is illustrated in Fig. 1. The data are corrected to the absolute number of circulating lymphocytes since granulocytosis occurs progressively over the course of bearing these tumors. PBL from normal mice show little or no detectable [^{3}H]TdR incorporation when cultured in the presence of solubilized tumor preparations. Two general patterns of dose-response kinetics were observed. The most prevalent pattern (Fig. 1 *a*) resembled closely

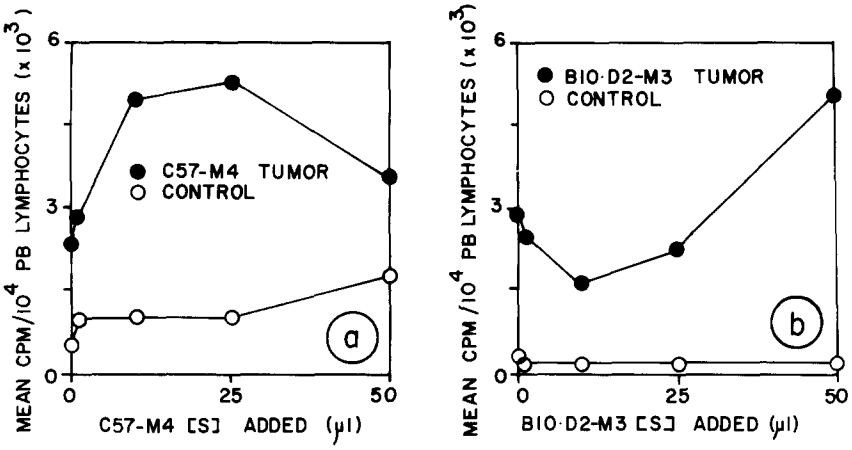

FIG. 1. Dose-response relationship of PBL from normal and tumor-bearing mice induced by added soluble homologous tumor antigen. The results are corrected to the absolute lymphocyte count. (*a*) C57-M4[s] on PBL from normal or C57-M4-bearing C57BL/6 mice, 14 days after tumor inoculation. (*b*) B10·D2-M3[s] on PBL from normal or B10·D2-M3-bearing C57BL/10 D2 mice, 21 days after tumor inoculation.

the responses to the T-cell mitogens, phytohemagglutinin and concanavalin. A, and to alloantigens; i.e., stimulation to a peak response followed by inhibition at higher antigen to mitogen concentrations (8, 14–16). This pattern was seen uniformly in the first 7–18 days after tumor inoculation. A less common pattern was observed later in the course of tumor bearing. Thymidine incorporation was inhibited below elevated background levels at very low antigen concentrations, but proliferation was stimulated at higher concentrations. This is illustrated in Fig. 1 *b*.

Interpretation of dose-response studies involving spleen and LN cell proliferation must account for two- to fivefold increases in total cell numbers in both cell masses during the natural history of bearing MCA tumors (9, 14–16). Data from a single experiment involving spleen cells are calculated both per unit cell number and per cell mass, as illustrated in Fig. 2. All subsequent data involving both spleen and LN cells are presented in terms of the respective total cell mass.

Spleen cells from normal animals proliferated slightly but detectably in some instances when solubilized homologous tumor was added at higher dose levels. As reported previously, spleen cells from tumor-bearing mice have high levels of background [^{3}H]TdR incorporation (8, 14). Two patterns of dose-response kinetics were also observed when antigen was added to spleen cells of such animals (Fig. 2 *b* and *c*). The biphasic pattern involving low-dose inhibition was more common late in the course of tumor bearing.

LN cells from tumor-bearing animals also gave vigorous proliferative responses to the solubilized homologous tumor preparation (Fig. 3). LN cells from normal mice did not proliferate significantly at any added concentration of solubilized tumor antigen. The physical proximity of the LN cell mass to tumor—whether regional, nonregional, or remote—was an important variable. The earliest proliferative responses were in the nodes regional to the tumor; later nonregional

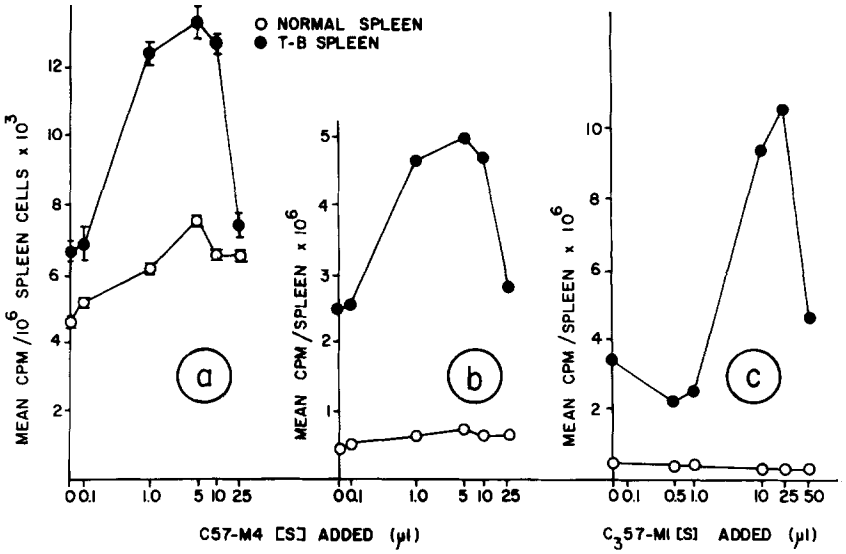

FIG. 2. Dose-response relationship of spleen cells from normal and tumor-bearing mice induced by soluble homologous tumor antigen. (*a*) Data expressed as cpm/10^6 spleen cells, 14 days after inoculation with C57-M4 tumor. (*b*) Same data shown in 2 *a*, expressed as cpm/spleen. (*c*) Stimulation of spleen cells by $C_3$57-M1[s] expressed in cpm/spleen 20 days after inoculation with $C_3$57-M1 tumor.

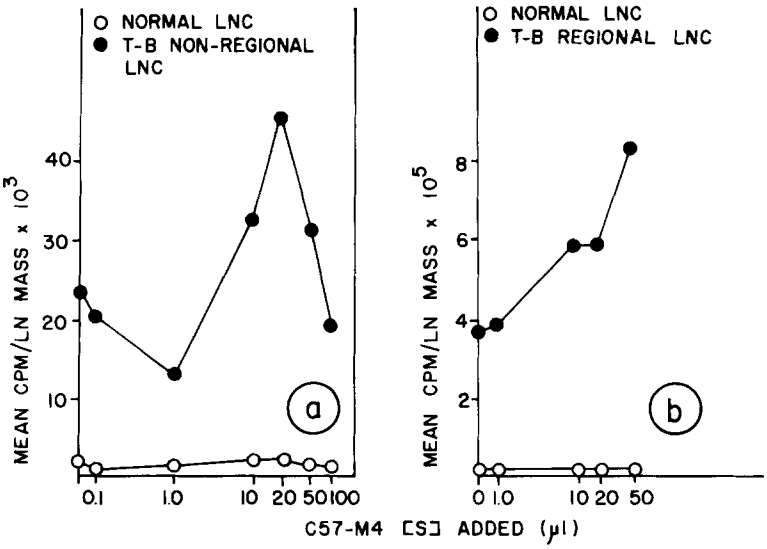

FIG. 3. Dose-response relationship of LN cells from normal and C57-M4-bearing C57BL/6 mice by the homologous antigen C57-M4[s]. (*a*) LN nonregional to tumor 28 days after tumor inoculation. (*b*) LN regional to tumor 25 days after tumor inoculation.

node masses were stimulated by homologous tumor antigen. As was found in the case of spleen cells and PBL, a biphasic dose-response pattern also occurred in LN cells taken from tumor-bearing animals, particularly in the nonregional node masses. The inhibition pattern was infrequent in regional LN-cell masses.

A highly significant positive correlation exists between the level of background [^{3}H]TdR incorporation in tumor-bearing animals' spleen cells and the peak level of incorporation stimulated by the homologous soluble tumor-antigen preparation, as illustrated in Fig. 4, and was true at each interval examined. The correlation was clearer but less consistent for LN cells, and seemed not to apply to PBL.

Changes in Patterns of In Vitro Proliferative Responses to Soluble Preparations of Homologous Tumor in Tumor-Bearing Animals as a Function of Interval After Tumor Inoculation. Proliferative responses to soluble tumor antigens

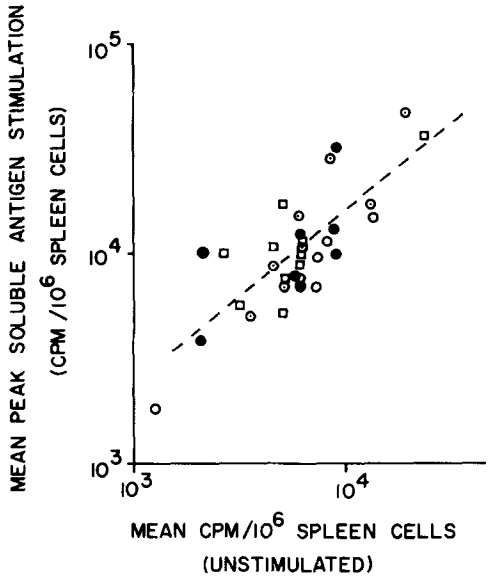

FIG. 4. Relationship between the incorporation of the peak value of unstimulated [^{3}H]TdR incorporation (background) in spleen cells taken from C57-M4 tumor-bearing C57BL/6 mice to peak [^{3}H]TdR incorporation in the same spleen cell cultures to which graded concentrations of C57-M4[s] was added. The spleen cells were taken at intervals of 7 (○), 14 (●), 21 (⊙), or 28+ (□) days after tumor inoculation. The line of regression (---) has a value of $r = 0.81$, and slope 1.59.

were examined in PBL, regional and nonregional LN, and spleen cells taken at varying times during the natural history of bearing 30 different MCA tumors in six mouse strains. Data illustrative of the findings in most experiments are given in Figs. 5 and 6.

Proliferative responses to the homologous antigen occurred earliest in LN regional to the site of tumor inoculation, as early as 7 days in some experiments. Levels of response in the regional nodes were maximal at 14–22 days and declined thereafter. The LN masses nonregional to the tumor site gave evidence of stimulation later in the course of tumor bearing (14–25 days) and the decline, which was regularly observed in regional nodes, did not occur.

Stimulation of proliferation in PBL by the homologous tumor antigen was detected as early as 10 days, and remained detectable at least 4–5 wk after tumor inoculation. The level of proliferation assessed by [^{3}H]TdR incorporation

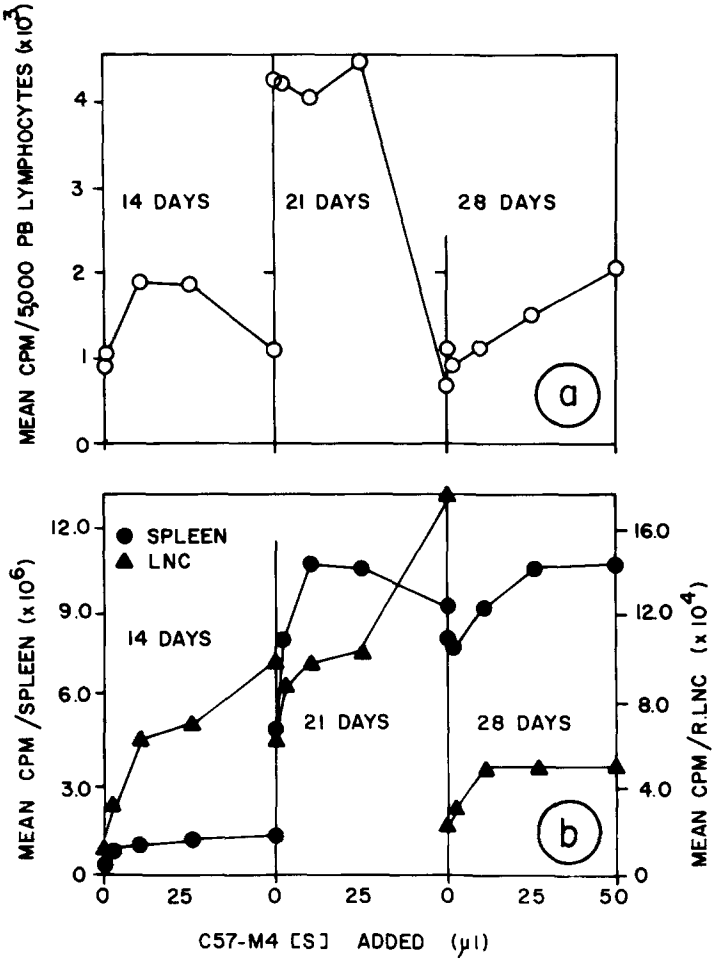

FIG. 5. Dose-response relationship to homologous tumor antigen of PBL, LN, and spleen cells from C57-M4 tumor-bearing C57BL/6 mice 14, 21, and 28 days after tumor inoculation. (*a*) PBL. (*b*) Spleen and LN cells.

increased to maximum values at 14–21 days and usually waned thereafter. Proliferation of PBL in response to any antigenic preparation was frequently reduced or undetectable late in the tumor-bearing period (30–40 days). Proliferative responses of spleen cells to homologous antigen were detected after that in regional node cells, but usually persisted throughout the course of tumor bearing, and at a level increasing with time (Fig. 5).

These data, together with those derived from experiments involving 30 other tumors, permit limited statements on the natural evolution of soluble antigen-evoked proliferative responses in the tumor-bearing mouse. The pattern is in many respects similar to responses evoked to antigenic stimulation from other sources; i.e., earliest in regional LN, followed by more generalized distribution of responsive cells to PBL, spleen, and distant LN (17).

Proliferative Responses to Homologous Tumor Preparations of Spleen and LN Cells Taken after Specific Tumor Immunization. The data described above

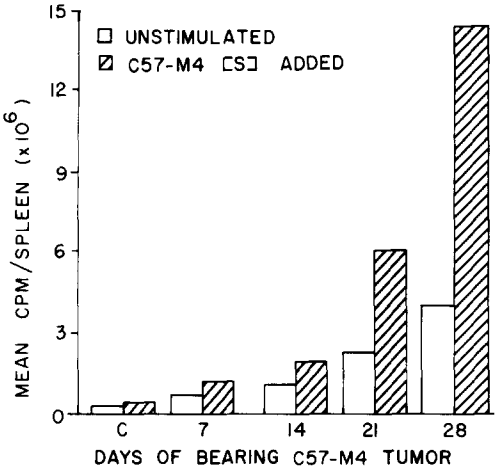

FIG. 6. Peak [^{3}H]TdR incorporation levels taken from dose-response curves, in spleen cells taken from normal or C57-M4 tumor-bearing C57BL/6 mice at 7, 14, 21, and 28 days after tumor inoculation.

pertain only to the soluble antigen-stimulated proliferative behavior in vitro of cells taken from tumor-bearing animals. Parallel studies were made of changes in homologous antigen-induced proliferation of LN and spleen cells after both tumor excision and soluble antigen immunization. The immunizing procedures used were those which induced resistance to homologous tumor transplantation (Table II). The results obtained were similar quantitatively to those in tumor-bearing hosts; LN cells showed a high level of stimulated proliferation with added homologous tumor preparations when assayed 14–30 days postexcision (Tables III and V). Spleen cell responses were also strong, although proportionally less than LN cells, in terms of increment of incorporation over background. No significant correlation was observed between background incorporation levels and stimulated incorporation after either type of immunization.

Evidence for Immunologic Specificity of Homologous Tumor Antigen-Induced Lymphocyte Proliferation. A key issue in interpreting the significance of the demonstrated in vitro proliferation of lymphoid cells after addition of solubilized tumor is that of immunologic specificity. Does the soluble tumor preparation stimulate proliferation in a general class of cells as a mitogen, or by triggering proliferation in a lymphoid memory cell subset as a consequence of specific receptor-antigenic ligand interaction? Evidence favoring the latter interpretation was obtained through three experimental approaches, as well as in the data to be described in a following section.

First, it was established that the tumors from which the antigen preparations were solubilized were indeed immunogenic, although they varied widely in capacity to elicit augmented resistance to challenge in the classic transplantation test model (11) (Table II). Resistance to challenge was highly specific to the homologous tumor in each instance (data not given), as has been firmly established in this model (1–3).

TABLE II

*Resistance of C57BL/6 Mice to Homologous Syngeneic Tumor Challenge Induced by Tumor Bearing and Surgical Excision**

Tumor designation	LD_{50} of challenge tumor cells‡	
	Control (limb excision only)	Tumor bearing and surgical excision of tumor
C57-M2	3.2	>6.0
$C_3$57-M1	<2.0	>6.0
C57-M4	<2.0	5.2
C57-M3	<2.0	4.6
$C_3$57-M2	<2.0	3.6

* Groups of C57BL/6 mice were injected s.c. in the hind limb with 1.0×10^5 cells from the indicated syngeneic MCA tumors, and the tumor-bearing limb excised surgically 7 days later. Groups of control mice were subject to limb excision at the same time. 7 days after excision $\log_{10}$ increments of the homologous tumor were injected s.c. on the opposite side in groups of 5–7 animals; tumor growth and lethality were determined. No detectable resistance was evoked toward any syngeneic nonhomologous tumor and these data are not shown.

‡ LD_{50} of tumor challenge calculated by method of Reed and Muench, given as log of the number of cells resulting in 50% mortality.

TABLE III

*Proliferative Responses to Various Syngeneic Solubilized Tumor Antigens in Regional LN Cells Taken 30 Days After Surgical Excision of C57-M4 Tumor in C57BL/6 Mice**

Syngeneic tumor antigen added	Peak [^{3}H]TdR incorporation by cell source‡	
	Controls	C57-M4[s] tumor immunized
	mean cpm/LN cell mass ± SE × 10^5	
None	0.04 ± 0.005	3.8 ± 0.02
C57-M2	0.06 ± 0.002	6.0 ± 0.60
C57-M3	0.06 ± 0.002	4.4 ± 0.30
C57-M4	0.08 ± 0.007	12.0 ± 0.24
$C_3$57-M1	0.08 ± 0.004	4.6 ± 0.30
$C_3$57-M2	0.04 ± 0.003	3.6 ± 0.28

* Assays were performed on pools of regional and nonregional LN cells taken 30 days after surgical amputation of the hind limb containing a C57-M4 tumor which had been inoculated (1×10^5 cells s.c.) 7 days before excision. Control groups were concurrently subjected to hind limb excision.

‡ Peak stimulation values ($\times 10^5$) taken from dose-response titrations, calculated in terms of the total LN mass examined.

Secondly, experiments were performed in which tumors were amputated after growth had been established, and an interval of 15–30 days had elapsed. This eliminated tumor recurrence and permitted the general effects of tumor-bearing per se to wane or disappear (8, 14). The effects of homologous solubilized tumor

preparations were assayed on LN and spleen cells taken from such animals. These data, discussed in the previous section, suggest that memory cells capable of responding to the tumor antigen at 30 days are present in highest concentration in the regional LN mass.

The third approach involved was to determine whether the solubilized tumor preparations were actually immunogenic in syngeneic mice and whether immunogenicity correlated with capacity to elicit in vitro proliferative reactions. Resistance to tumor challenge was regularly elicited in animals immunized by injecting solubilized tumor preparation in FCA over a 40 day period, then challenging with incremental numbers of homologous living tumor cells (Tables IV and V). In vitro stimulation of LN and spleen cells with homologous tumor antigens was regularly elicited in these circumstances.

TABLE IV

*Resistance of C57BL/6 Mice to Homologous and Syngeneic Tumor Challenge Induced by Immunization with Solubilized Tumor Preparations**

Tumor used to challenge after immunizing procedure	LD_{50} of challenge tumor cells, in groups immunized with indicated tumor antigen preparation‡					
	Control§	C57-M2[s]	$C_3$57-M2[s]	C57-M3[s]	$C_3$57-M1[s]	C57-M4[s]
C57-M2	<3.0	4.5	3.6	3.5	3.0	<3.0
C57-M3	<3.0	<3.0	<3.0	3.6	<3.0	<3.0
C57-M4	<3.0	<3.0	<3.0	<3.0	<3.0	3.4
C57-M1	<3.0	<3.0	<3.0	<3.0	3.6	4.0

* Groups of C57BL/6 mice were injected i.m. with 0.1 mg of the indicated solubilized tumor preparation, incorporated in FCA; 28 days later, the groups were reinjected s.c., with the identical preparations. 7 days after this, the groups were injected i.m. with $\log_{10}$ incremental numbers of cells from the indicated tumor, and tumor growth and mortality recorded.

‡ LD_{50} calculated by the Reed-Muench method, given as the log of the number of cells eliciting 50% mortality. The values of 3.0×10^6 or more represent significant resistance over control values.

§ Control mice received identical injections of FCA with RPMI-1640 media.

The results of each approach were consistent, therefore, with the interpretation that the stimulatory capacity of the solubilized tumor preparations reflect specific responses in receptor-bearing cell subsets. Moreover, in the experiments used to illustrate the point, tumors of the series C57-M1 through $C_3$57-M3, which were most immunogenic in vivo, yielded antigenic preparations most stimulatory to cells from tumor-bearing or tumor-immune animals in vitro (C57-M2 and C57-M4). Those least evocative of in vivo resistant were least stimulatory ($C_3$57-M2 and C57-M3) in vitro. Removal of the tumor by amputation gave augmented rather than reduced responses in vitro, as compared with those of tumor-bearing animals. This would appear to exclude a mitogenic effect on a susceptible proliferating cell subpopulation peculiar to the tumor-bearing state. The data appear to justify the use of the term "tumor antigen" in relation to the solubilized tumor material.

Immune Responses to Multiple Syngeneic Tumor Antigens in the Tumor-

TABLE V

*Effect of Immunizing with Homologous or Syngeneic Solubilized Tumor Antigens in CFA on Proliferation Induced In Vitro by Solubilized Antigens, 45 days later**

Tumor antigen used to immunize source of spleen or LN cells	Mean peak [^{3}H]TdR incorporation values/cell mass ($\times 10^5$) by solubilized tumor antigen added in assay			
	None	C57-M2[s]	C57-M3[s]	C57-M4[s]
No tumor, FCA, and medium alone				
Spleen	0.40	0.38	0.61	0.91
LNC	0.05	0.04	0.04	0.11
C57-M2[s] in FCA				
Spleen	2.62	9.14	10.28	29.26
LNC	0.47	1.70	2.77	4.22
C47-M4[s] in FCA				
Spleen	1.94	4.94	7.50	25.97
LNC	0.30	1.36	1.07	2.64

* Groups of mice were injected i.m. with 0.1 mg of the indicated soluble tumor antigen in RPMI-1640, or medium alone in the case of controls in FCA. The injection was repeated 30 days later s.c., and spleen or LN cells assayed 15 days later for proliferative responses to solubilized syngeneic tumor antigens.

Bearing Mouse. It was apparent early in these studies that lymphoid cell proliferation was regularly elicited by preparations derived from syngeneic MCA tumors in addition to that induced by those from the homologous tumor. These so-called cross-reactions to solubilized tumor antigens have been studied in four different experimental contexts: First, responses of LN or spleen cells taken after tumor excision were examined in parallel with the in vivo challenge in which tumor-unique transplantation resistance was demonstrated. Proliferative responses of LN cells to various syngeneic tumor antigens are illustrated by that shown in Table III. The strongest responses in terms of [^{3}H]TdR incorporation were uniformly found with the homologous tumor preparation. Proliferation was also elicited by syngeneic tumor antigen preparations; this responsiveness to syngeneic tumor antigens was not paralleled by resistance to tumor transplantation.

Second, transplantation resistance and proliferative responses to multiple syngeneic antigens were assessed after active immunization with solubilized tumor antigen; spleen and LN cells were examined from animals, which had been immunized with solubilized antigens in FCA. Parallel in vivo challenges were made with living tumor cells. The results, as exemplified in Tables IV and V, were unexpected in that not only were proliferative responses elicited in LN and spleen cells against syngeneic tumor antigens, but in vivo cross-resistance was induced toward some syngeneic tumors. For example, C57-M2 was resisted by animals immunized with several preparations while others, C57-M3 and C57-M4, were resisted only by animals which were immunized with the respective homologous antigen preparation. This represents the first known evidence that transplantation immunity evoked by chemically induced fibrosarcomas in mice may be induced by shared or cross-reacting antigens.

Third, checkerboard assays of proliferative responses to multiple syngeneic antigen preparations by various lymphoid cell masses were made at a single time (20–33 days) after tumor inoculation from individual mice bearing one of several syngeneic tumors; peak incorporation values taken from dose-response titrations and calculated in terms of total cell mass are presented for exemplary experiments involving tumors in four sublines of C57BL/10 mice (Table VI). The data illustrate that extensive proliferative responses were elicited toward syngeneic tumor-antigen preparations. In this single-time, cross-section type of experiment, the responses to homologous tumors were not always the strongest ones. Some tumors appeared to evoke immune responses to more syngeneic tumor-antigen preparations than others. The data, therefore, do not support the occurrence of a shared tumor antigen, represented more or less equally in each member of a family of syngeneic tumors.

Fourth, checkerboard studies were made of different cell masses at varying intervals after tumor inoculation. The data, as exemplified by the experiments shown in Fig. 7, show that the so-called cross-reacting proliferative responses elicited by syngeneic tumor antigens are both time-course and cell-mass dependent. The most frequent pattern observed was that significant or strong proliferative responses to homologous tumor antigens appeared first in the regional LN and PBL at 10–14 days and later at 2–3 wk in spleen or nonregional LN masses. Proliferation in response to syngeneic tumor antigens appeared at 2–3 wk in the regional LN masses and reached maximal levels in spleen and nonregional LN masses at 3–4 wk. Homologous tumor-antigen responsiveness frequently was attenuated or absent in the regional LN at 3–4 wk at the same time responses to syngeneic tumors were significant at the same cell mass. The results of 14 similar experiments evaluating changes in temporal occurrence and cell-mass distribution of responses to syngeneic tumor antigens are summarized in Table VII.

Discussion

Solubilization of the tumor cells of MCA-induced fibrosarcomas gave preparations which induce tumor resistance in vivo and stimulate lymphoid cells taken from tumor-bearing or tumor-immune mice to proliferate in vitro. The assay developed to measure proliferative responses in vitro appears to reflect interactions between tumor antigens and immunocompetent cells. The evolution of cell proliferative responses in various lymphoid cell masses during the natural history of tumor bearing apparently involved accumulation of antigen responsive cells first in the regional LN; this is followed by spread to more remote lymphoid cell masses. These proliferative responses were initially strongest toward the homologous tumor-antigen complex, but strong responses to some but not all syngeneic tumor antigens were detected after 2–4 wk of tumor bearing. The time-course and cell-mass patterns of such "cross-reactions" are interpreted to indicate the existence of multiple antigenic components of the tumors, some of which are shared, others apparently not.

The assay procedure developed and used in these studies proved reliable and reproducible, although its several important limitations require stipulation.

TABLE VI

*Effect of Bearing Various Syngeneic Tumors for a 3 wk period Upon Solubilized Tumor Antigen-Induced Proliferation in Spleen Cells of Congenic Resistant C57BL/10 Mouse Sublines**

Subline and subline tumor inoculated	No. of spleen cells ($\times 10^6$)	Mean peak [^{3}H]TdR incorporation per spleen ($\times 10^6$), by origin of solubilized tumor antigen				
		No antigen added	Tumor antigen			
			B10-M1	B10-M2	B10-M3	B10-M4
C57BL/10						
B10 (no tumor)	75	0.24	0.39	0.61	0.68	0.57
B10-M1 (tumor)	151	1.43	1.48	2.02	2.58	2.42
B10-M5	169	1.50	2.40	2.00	3.81	3.21
B10-M2	170	3.06	3.69	2.47	4.72	3.61
B10-M4	181	1.50	2.24	1.91	3.99	3.36
B10-M3	270	5.96	8.06	6.99	14.41	11.86
			BR·C2-M1	BR·C2-M2	BR·C2-M3	
C57BL/10·Br						
B10·BR (no tumor)	100	0.20	1.80	0.90	0.60	
B10·BR·C2-M3	242	1.40	7.10	6.80	4.90	
B10·BR·C2-M1	258	2.30	5.30	4.60	3.40	
B10·BR·C2-M2	452	3.10	16.40	11.80	10.90	
			A·C2-M1	A·C3-M1	A·C3-M2	A·C3-M3
C57BL/10·A						
B10·A (no tumor)	98	0.21	0.24	0.45	0.37	0.21
B10·A·C2-M2	146	0.75	1.60	3.30	0.94	1.40
B10·A·C3-M3	155	0.70	0.70	1.10	0.57	0.63
B10·A·C3-M2	220	1.40	2.60	3.90	1.80	2.20
B10·A·C3-M1	268	3.50	5.80	10.60	3.90	3.50
B10·A·C2-M1	278	2.70	6.90	10.60	3.30	4.80
			D2-M1	D2-M2	M2-M3	
C57BL/10·D2						
B10·D2 (no tumor)	91	0.29	0.89	1.20	0.47	
B10·D2-M1	133	0.91	2.50	2.70	2.00	
B10·D2-M2	178	1.50	7.00	6.60	4.50	
B10·D2-M3	261	2.80	17.20	10.70	6.30	

* Tumor cells taken from third passage tumor induced by MCA in each C57BL/10 subline indicated were inoculated (1×10^6 cells s.c.) 20–23 days earlier, and quadriplicate cultures of spleen cells were incubated 3 days after adding various amounts (0.01–50 μl) of the indicated solubilized tumor antigen prepared from the homologous and other syngeneic tumors. Proliferation was assayed in terms of [^{3}H]TdR incorporation; peak mean values for incorporation taken from dose-response curves, were calculated as counts per minute per spleen.

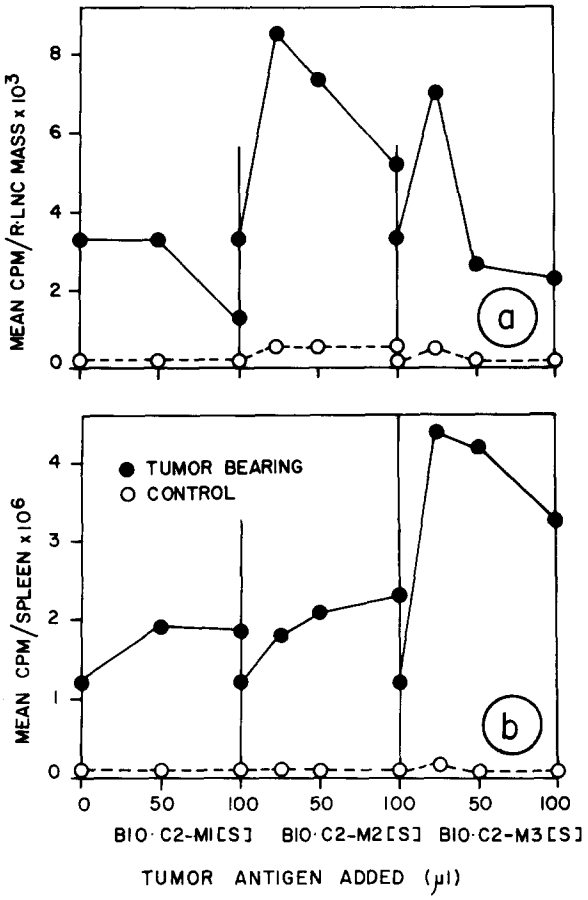

FIG. 7. Dose-response relationship between homologous (B10·C2-M3[s]) and syngenic (B10·C2-M1[s] and B10·C2-M2[s]), antigens of spleen and regional LN cells (R·LNC) from normal and B10·C2M3 tumor-bearing C57BL/10, 22 days after tumor inoculation. (*a*) Regional LN. (*b*) Spleen.

First, the stimulatory components of the solubilized tumor preparations have not been isolated and identified in such a way as to provide a quantitative basis for relating the antigen content of different preparations from the same tumor. Accordingly, it has been necessary to perform, in each test, dose-response titrations involving varying concentrations of each preparation over a 1,000-fold range. While this approach imposes logistical restraints upon the number of variables which can be explored in a single experiment, no alternative measure of antigen content seems completely appropriate at this time. Secondly, the activity in these preparations did not always survive freezing or lyophilization in terms of a reproducible capacity to stimulate equal levels of proliferation. However, the activity remained constant at 4°C for 3–4 wk. This required regular production of fresh preparations, and these varied somewhat in activity from batch to batch. Both of these limitations can probably be eliminated through use of semipurified preparations now under investigation (R. Blackstock, Schimpff, and Smith, unpublished data).

TABLE VII
Summary of Observed Patterns of Lymphoid Cell Stimulated by Solubilized Tumor Antigens, at Varying Intervals During Tumor Growth

Intervals after inoculation of tumor	Occurrence of maximal stimulation and specificity in terms of homologous syngeneic and syngeneic antigens, by cell mass			
	PBL	Regional lymph nodes	Nonregional lymph nodes	Spleen
days				
1–10	Detectable, homologous tumor antigen	Strong, homologous, tumor only	No stimulation	No stimulation
11–22	Strong, homologous, and syngeneic	Strong, homologous, and syngeneic	Strong, homologous, and syngeneic	Strong, homologous, and syngeneic
22–38	Weak or undetectable particularly in terminal stages	Weak or undetectable to homologous antigen; syngeneic antigens give variable stimulation	Strong, homologous and syngeneic, waning terminally	Strong homologous, and syngeneic
15–30 postsurgical excision of tumor	—	Strongest; homologous usually greatest, also detectable syngeneic	Moderate; homologous and syngeneic	Moderate; homologous and syngeneic

The dose-response assays revealed an as yet unexplained inhibition of proliferation at low antigen concentrations. This inhibitory activity reduced the high spontaneous "background incorporation" levels of [^{3}H]TdR, which are regularly encountered in spleen or LN cell masses taken from tumor-bearing animals, to lower levels approaching those characteristic of normal spleen or LN cells. This inhibitory effect occurred with syngeneic tumor preparations as well as homologous ones, and the pattern of time and cell mass involved was similar to that of the stimulatory response. The biphasic character of the inhibition-stimulation phenomenon could indicate either that the solubilized tumor preparation contains both inhibitory and stimulatory components affecting the same cells; or that the cell mixtures assayed include both proliferating cell subsets, which are subject to inhibition, and other subsets which are subject to stimulation. The data do not yet permit any firm conclusion with respect to the nature of significance of the inhibitory phenomenon in this model.

Baldwin et al. (18–20) have also described specific inhibition of in vitro lymphocyte-mediated tumor cytotoxicity by addition of solubilized tumor antigen. The low-dose inhibition observed appears, however, distinctive from that reported by Baldwin et al. and the inhibition which occurs in this system when amounts of antigen are added above those

which induce optimal stimulation proliferation in this system, or which occurs when serum from a tumor-bearing animal is added, the so-called blocking effect (Forbes, Calderwood, and Smith, unpublished data, and others [references 21–23]).

The most likely explanation of the significant positive correlation between the background level of [^{3}H]TdR incorporation by spleen cells from tumor-bearing animals and antigen-stimulated incorporation is that the same cell subsets are responding in vivo to antigen-driven proliferation and in vitro to added antigen (24–32). The larger the subset size, the higher the level of in vivo and in vitro proliferation. On the other hand, it is possible that the in vivo proliferation indicated by high level spontaneous proliferation may provide triggering or augmenting influence which permits effective responses to the added antigens in vitro. An analogous effect has been described recently in experiments showing a synergistic or triggering action of alloantigen responsive cells upon susceptibility to B-cell mitogens (33).

The assay data reflect proliferative activity which correlates with the in vivo immunogenicity of the tumor or its soluble derivative. It has not been correlated with data obtained by assay techniques which depend upon cell-mediated cytotoxicity (6, 7), colony inhibition (4, 5), or lymphokine production (34–36) as end points. It appears to complement data obtained by these techniques in several ways. Because DNA synthesis measured in the assay is related to the number of proliferating cells, it has the potential of permitting estimation of the original subset size of a responding lymphoid cell subpopulation. Moreover, the possibility exists of examining such subsets for individual specificity toward components of the tumor-antigen complex through techniques for eliminating one proliferating subset and testing for residual responding cells, as has been done in other contexts (37–40).

Lymphoid cell proliferation occurred in response to antigens derived from some but not all syngeneic tumors, whether taken from a tumor-bearing or tumor-immune animal. This differs somewhat from the reported results in other in vitro assay systems. Most in vitro studies have revealed nearly universal cross-reactivity between chemically induced (41–44) or spontaneous (43) tumors to the same histogenetic type (41), and those induced by the same oncogenic viruses (44). The patterns of cross-reactions observed in the MCA-induced tumors examined here are most consistent with immunologic responses to multiple shared or unshared specificities on each tumor rather than responses toward a single, broadly shared tumor-associated antigen. The responses to the soluble antigen preparations as immunogens, the time-course of lymphoid cell responses, and the pattern of spread throughout the various cell masses, each suggest multiple independent subset responses to tumor-born antigenic structures.

These antigens may reflect quantitative differences in cell surface expression of a large number of normal structures, as well as qualitative differences related to oncogenic events. Such structures could include any or all of the following: virus related membrane components (44–46), cell differentiation associated or embryonic structures (41–49), membrane structures detected as histocompatibility antigens (44, 50–53), or unique structures formed by concatenations of any of these as a tumor-specific matrix as suggested by Boyse and Old (54, 55). That each specificity is related to tumor-specific transplantation rejection is not necessarily required by such a hypothesis. For example, homologous tumor cells would be subject to in vivo immunologic attack by cytotoxic cells or antibody elicited by each antigenic structure of the array of specificities on that tumor. A syngeneic tumor, on the other hand, would be exposed in vivo only to those potentially destructive elements of the immune response directed toward shared structures. Such quantitative differences could conceivably account for tumor-unique rejection of homologous tumors while syngeneic tumor implants survive, since in the transplantation model a growth or no growth end point is assessed.

An important and possibly practical implication of the data presented is that in order to assess tumor-specific immunity in tumor-bearing man or animals, consideration must be given to multiple variables. These include the interval after tumor inoculation or development, the lymphoid cell mass examined, and the extent of multiple specificities defined. Single time-frame or cell-mass sampling, such as examination of PBL only, does not necessarily reflect the broad immunologic committment of the individual to tumor-borne antigenic specificities or the cellular ecology of this committment. These variables have been largely unaccounted for in most previous work aimed at defining tumor-specific immunity. The total "cell ecology" approach appears essential to interpreting current studies aimed at accounting tumor responsive lymphoid cells in terms of responding cell class or subclass.

Summary

Experiments were designed to explore the apparent paradox that methylcholanthrene-induced tumors of mice evoke tumor-unique transplantation immunity but reveal almost complete cross-reacting antigenicity in tests of lymphocyte behavior in vitro. The approach involved use of tumor membranes solubilized in 3 M KCl, employed both as the stimulating antigen source in a new in vitro proliferation assay of lymphocyte recognition, and as immunogens in vivo. The kinetics of the assay resembled those of in vitro tests of mitogen or specific antigen stimulation in other systems. Lymphoid cell proliferation was assessed in peripheral blood leukocytes, lymph nodes (LN), and spleen over the course of tumor bearing, and in animals immunized by tumor amputation or with the solubilized antigens. The pattern of spread of reactivity was from regional LN to spleen, peripheral blood, and nonregional nodes in each circumstance. An unexplained low antigen dose inhibitory phenomenon was encountered in spontaneously proliferating cell subpopulations taken from some tumor-bearing animals. In vitro responses to some but not all solubilized antigens made from multiple syngeneic tumors were detected in each circumstance. The soluble antigens also induced shared resistance to some tumors. The patterns of spread of responsiveness to syngeneic tumor antigens, the time-course, and relative intensity were most compatible with independent clonal responses to multiple tumor-borne antigens, some but not all of which are shared in any family of syngeneic tumors.

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